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Hormonal induction of the secretory immune system in the mammary gland

(lymphocyte homing/IgA/lactation/gut-associated lymphoid tissue)

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ABSTRACT **The secretory immune system of the mammary gland is undeveloped in virgin mice but becomes active at term and during lactation. This change appears to depend on migration to the mammary gland of precursors of IgA-secreting cells derived from the gut-associated lymphoid tissue, an origin which explains the specificity of milk IgA antibodies for enteric organisms. Because development of the epithelial components of the mammary gland is clearly under hormonal control, we examined the effect of mammotropic hormones on differentiation of the immune elements. Under a combined regimen of progesterone, estrogen, and prolactin, development of the glandular epithelium occurs with concomitant increments in the number of IgA-secreting plasma cells and amount of intraepithelial IgA. These increases appear to be due to enhanced capacity of the gland to attract or retain precursors of IgA plasma cells derived from gut-associated lymphoid tissue. Testosterone, which antagonizes lactation, also antagonizes development of the secretory immune system and decreases cellular trapping in the lactating gland. The ability of the gland to trap IgA immunoblasts is probably contingent upon a hormone-induced increase in receptors.**

Antibodies in maternal milk can play an important role in protecting infants against gastrointestinal infections. Suckling of infants therefore is thought to be particularly desirable in underdeveloped countries where such infections are especially common. The major antibody class found in milk, as in the other exocrine secretions of most mammalian species, is IgA which is thought to be derived from local plasma cells and transported across the epithelium to the milk. A question arises as to how the population of plasma cells residing in the mammary gland acquires specificity for gastrointestinal organisms. We have studied this problem in a mouse model (1, 2).

In the mouse mammary gland, the number of plasma cells synthesizing IgA and the amount of intraepithelial IgA increase dramatically as lactation becomes established and they decline after weaning (1). The increase in IgA plasma cells is not accompanied by equivalent increases in plasma cells making other classes of immunoglobulin. By cell transfer studies (2) we have been able to show that the onset of the increase in IgA plasma cells in the mammary gland late in pregnancy is closely related in time to the onset of an enhancement in the capacity of the mammary gland to attract or retain IgA-bearing immunoblasts derived from the gut-associated lymphoid tissue (GALT). This enhancement in the attractiveness of mammary gland tissue for GALT-derived immunoblasts persists throughout lactation and declines after weaning. Immunoblasts derived from peripheral nodes (PN) do not show the tendency of GALT immunoblasts to migrate to lactating mammary gland. Our observations can thus explain the specificity of milk antibodies for gastrointestinal organisms: the antibodies are produced by plasma cells derived from precursors originally stimulated by antigens in the GALT.

A second question arises as to what controls the increase in mammary gland plasma cells late in pregnancy and during lactation. One possibility is that the controls are hormonal. An analysis of this idea is the subject of the present paper. Because differentiation of the epithelial part of the mammary gland is hormone-dependent, we investigated whether exogenous hormones administered to virgin mice can also effect the morphological and functional changes in the immune system of the mammary gland that are observed during natural pregnancy and lactation. Estrogen, progesterone, prolactin, and cortisone, known to promote development and lactation (3), were investigated alone or in combination in virgin mice. In addition, testosterone was studied because of its inhibitory effects on mammary gland development. We found that the development of the secretory immune system, assessed in terms of IgA plasma cells and intraepithelial IgA, parallels general glandular development and depends on the combined effect of estrogen, progesterone, and prolactin. Furthermore, this combination of hormones also induces the mammary gland of virgin females to act as a homing site for IgA immunoblasts taken from mesenteric lymph nodes (MN), a phenomenon that can be demonstrated under natural conditions only late in pregnancy and during lactation (2). In contrast, testosterone administered to lactating mothers promotes involution of the gland and prevents the increase in IgA plasma cells that is ordinarily seen. Moreover, the MN precursors of IgA plasma cells fail to home to mammary glands of testosterone-treated mice.

MATERIALS AND METHODS

Mice. Male or female CAF_1/J mice (The Jackson Laboratory, Bar Harbor, ME) were used at 6–8 weeks of age. Males were surgically castrated under sodium pentothal anesthesia; treatment with hormones was begun 2 weeks later.

Hormones. Progesterone, estrogen (β-estradiol-3-benzoate), testosterone, cortisone, and ovine prolactin (23 international units/mg) were obtained from Sigma Laboratories (St. Louis, MO). Sterile olive oil was used as a vehicle for the lipid-soluble hormones; prolactin was dissolved in sterile saline. Schedules for treatment, adapted from refs. 3–6, are shown in Fig. 1. Five or more animals were injected subcutaneously with 0.1 ml of the indicated dose of each preparation daily and sacrificed on

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Abbreviations: GALT, gut-associated lymphoid tissue; MN, mesenteric lymph nodes; PN, peripheral lymph nodes; PI, preference index; ^{125}IUDR, [^{125}I]iododeoxyuridine.

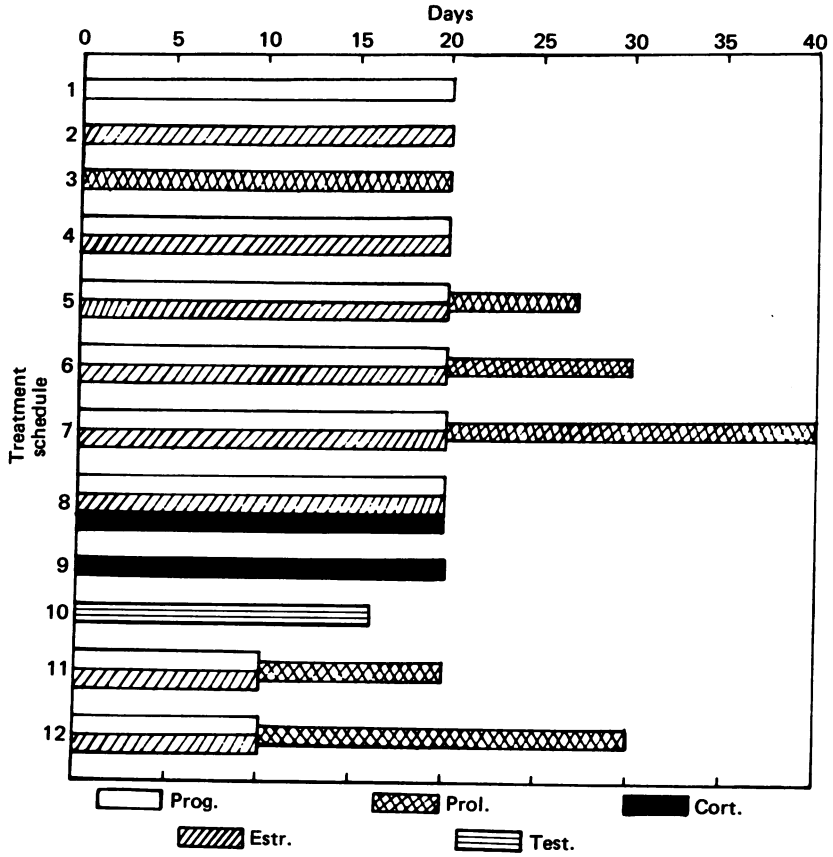

FIG. 1. Schedules of hormone treatments. Prog, progesterone, 5 mg; Prol, prolactin, 2 mg; Cort., cortisone, 1 mg; Estr., estrogen, 4 μg; Test., testosterone, 5 mg.

the day following the last injection. Controls were injected with olive oil or saline alone.

Immunofluorescence. Mammary glands were examined by immunofluorescence and by standard histological techniques as described (1). Acetone-fixed frozen sections were stained with fluoresceinated antisera to mouse IgA, IgM, or IgG (Meloy Laboratories, Springfield, VA). Each section was evaluated for the extent of glandular development, intraepithelial immunoglobulins, and number of plasma cells. Plasma cells were counted in 20 fields with a ×25 objective. The general histologic appearance was assessed by light microscopy with hematoxylin and eosin and methyl green/pyronin stains.

Cell Transfer and Recovery. Labeling of DNA-synthesizing cells and their transfer and recovery have been described (2). In brief, lymphocytes in suspension were labeled in the presence of 2 μCi of [^{125}I]iododeoxyuridine (^{125}IUDR; Amersham/Searle, Arlington Heights, IL) per ml for 90 min at 37°. About 10^7 cells (>90% viable by trypan blue dye exclusion) were injected intravenously per recipient. Radioactivity in recipients' tissues was measured in a Nuclear Chicago gamma counter.

RESULTS

Effect of Exogenous Hormones on Development of the Mammary Gland and Its Secretory Immune System. Mammary glands of normal male mice contained only sparse ductules and adipose tissue. In virgin females, a few acini were also seen. Although the epithelium in both sexes was practically devoid of immunoglobulin, occasional IgA plasma cells were seen in the stroma; IgM and IgG plasma cells were rare. Mammary glands of mice treated with olive oil alone (or saline alone) were similar to those of untreated virgin animals.

To study the effect of exogenous hormones, virgin females were treated according to one of the schedules in Fig. 1. Histological changes in mammary glands from normal virgin, normal lactating, and hormone-treated mice are illustrated in Fig. 2. Treatment with any of the hormones alone, or with progesterone and estrogen combined, caused minimal to moderate increases in number of acini and ducts and moderate increases in the number of IgA plasma cells (Table 1). The greatest induction of development caused by an individual hormone was observed after prolactin treatment. In terms of epithelial development, intraepithelial IgA content, and number of IgA plasma cells, combined treatment with progesterone, estrogen, and prolactin (groups 7–9; Fig. 2 *E* and *F*) was the most effective. The presence of intraepithelial IgA indicates that the normal functional relationship between plasma cells and epithelial cells for transport of IgA, which is so characteristic of the secretory IgA system, was achieved.

Full development of the mammary gland as seen in 20-day naturally lactating mice (Table 1, group 13; Fig. 2 *C* and *D*) was not observed. Nevertheless, the number of IgA plasma cells per unit area of tissue in virgins treated with progesterone, estrogen, and prolactin (groups 7–9) compared favorably with the number seen in naturally pregnant mice at term (group 12). Suckling of virgin, hormone-treated females (schedule 5) by foster litters (days 21–28) stimulated milk production but did not further increase the IgA plasma cell population. Cortisone, secondarily associated with mammary gland development (6), at best slightly augmented the effect of progesterone and estrogen (Table 1, groups 6 and 10). IgG and IgM plasma cells remained sparse in all groups, and the intraepithelial content of these immunoglobulins was also negligible.

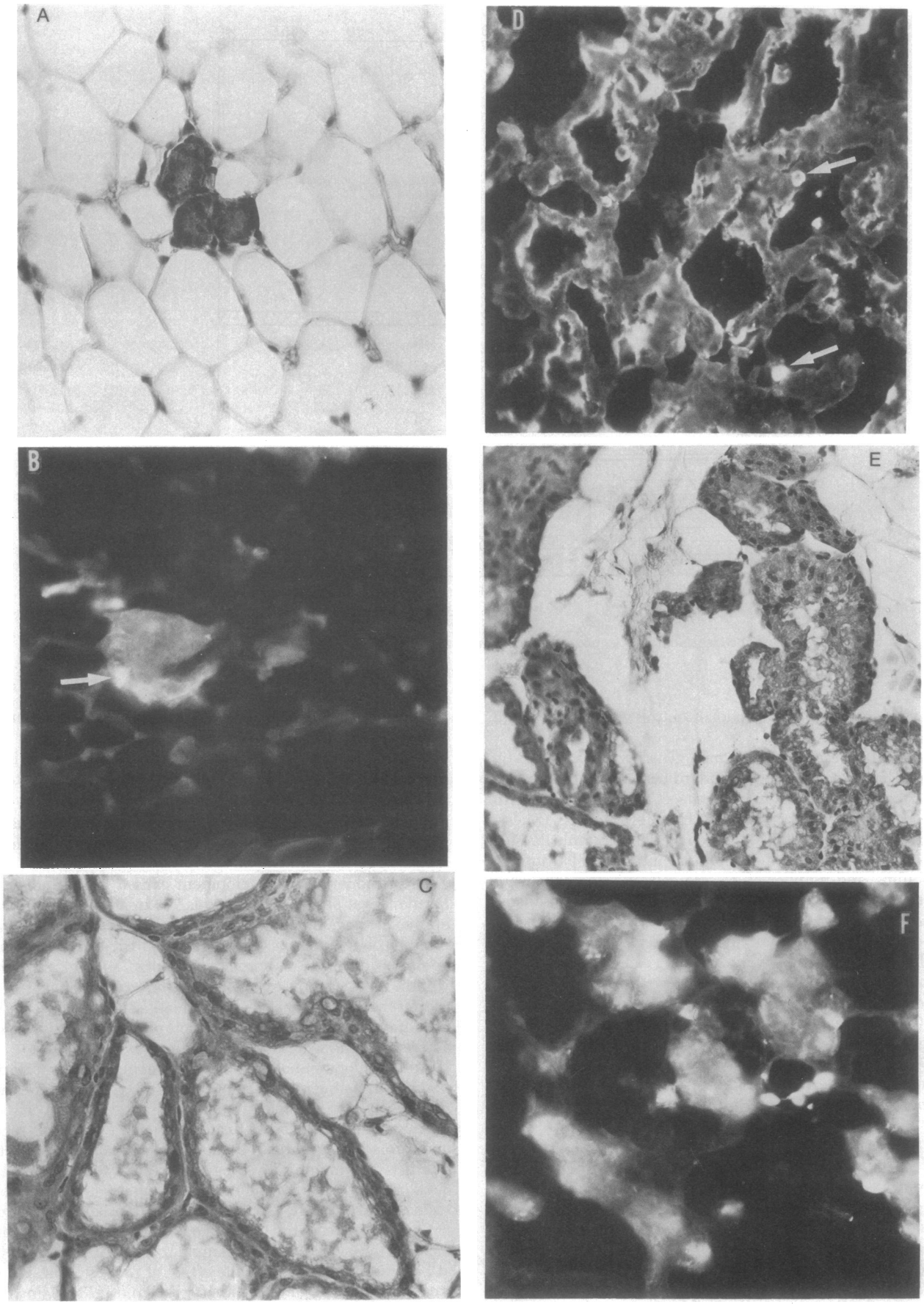

FIG. 2. Paraffin sections of female mouse mammary glands stained with hematoxylin and eosin (*A*, *C*, and *E*) or frozen sections stained with fluoresceinated anti-IgA (*B*, *D*, and *F*). (×240.) (*A*) Untreated 8-week-old virgin, showing adipose tissue and occasional ducts (center).

Table 1. Effects of various hormone treatments* on number of IgA plasma cells in mammary gland

Group	Mice†	Sex	Treatment schedule*	Number of cells‡
1 & 2	Vir.	F	None or oil vehicle	24 ± 5
3	Vir.	F	1, Prog.	60 ± 15
4	Vir.	F	2, Estr.	70 ± 23
5	Vir.	F	3, Prol.	120 ± 12
6	Vir.	F	4, Prog. + Estr.	100 ± 7
7	Vir.	F	5, Prog. + Estr.; Prol.	200 ± 20
8	Vir.	F	6, Prog. + Estr.; Prol.	210 ± 25
9	Vir.	F	7, Prog. + Estr.; Prol.	220 ± 30
10	Vir.	F	8, Prog. + Estr. + Cort.	130 ± 18
11	Vir.	F	9, Cort.	40 ± 10
12	TP	F	None	175 ± 50
13	L	F	None	1050 ± 100
14	L	F	10, Test.§	200 ± 16
15	N	M	None	10 ± 2
16	N	M	Oil vehicle	12 ± 3
17	C	M	None	32 ± 6
18	N	M	5, Prog. + Estr.; Prol.	20 ± 3
19	C	M	5, Prog. + Estr.; Prol.	120 ± 23

* See Fig. 1. Prog., progesterone; Estr., estrogen; Prol., prolactin; Cort., cortisone; Test., testosterone.
† Vir., virgin; TP, term pregnant; L, lactating, 20 days; N, normal; C, castrated.
‡ Mean (±SD) of IgA plasma cells in 20 fields (×25 objective); one section of each mammary gland area from each of at least five mice was examined.
§ Testosterone was begun 4 days post partum.

Combined hormone treatment was essentially without effect in intact males (group 18). However, castrated males that received the triple treatment (group 19) responded with marked glandular development as well as pronounced increases in number of IgA plasma cells. This result suggests that androgens inhibit the development of the secretory immune system in the mammary gland. To investigate this possibility further, testosterone was administered to lactating females. This treatment produced an arrest of mammary gland development, a concomitant reduction in plasma cell numbers, and a decrease in intraepithelial IgA (group 14).

Influence of Hormones on Homing of MN Lymphoblasts to Mammary Glands of Virgin Mice. We have presented evidence (2) that immunoblasts derived from MN home to the mammary glands of near-term and lactating mice but not to those of normal virgin mice. Immunoblasts derived from PN do not share this property. The homing cells bear surface IgA, not IgG or IgM, and can be identified as IgA plasmablasts in the mammary gland within 24 hr of transfer. The comparative efficiency with which MN blasts, relative to PN blasts, seek the mammary gland parallels the degree of development of the mammary gland and is conveniently expressed as a preference index (PI) defined as

$$\text{PI} = \frac{\%\text{ injected radioactivity/g of mammary gland in MN recipients}}{\%\text{ injected radioactivity/g of mammary gland in PN recipients}}$$

Table 2. Effect of progesterone, estrogen, and prolactin* on homing of ^{125}IUDR-labeled MN cells to mammary glands of virgin mice

Donor cells	Label recovered in mammary gland† %/g	%/organ	PI‡
MN	0.50 ± 0.02	1.80 ± 0.03	2.8
PN	0.18 ± 0.15	0.75 ± 0.40	

The experiment shown in the table is one of a total of seven. The mean (±SEM) PI for the seven experiments was 2.4 ± 0.5.
* Hormone treatment schedule 12 (Fig. 1).
† Percentage of total label injected (mean ± SD, three animals), recovered one day later.
‡ Preference index (see text).

Because exogenous hormones produce morphological changes in the secretory immune system of the mammary gland like those occurring naturally during pregnancy and lactation, we tested the most effective hormone combination for its ability to prepare mammary glands of virgin mice to act as homing sites for the GALT precursors of IgA plasma cells. MN and PN cells, from normal virgin females, were labeled in S phase with ^{125}IUDR *in vitro*, and their migration was compared after intravenous injection 1 day following termination of hormone treatment. One representative experiment, of a total of seven, is presented in Table 2. A PI of 1 indicates no difference in the behavior of the two donor populations. PI >1 reflects a greater tendency on the part of MN cells than PN cells to go to the mammary glands. As previously shown (2), in five experiments in which migration in normal virgin or postlactating mice was studied, PIs ranged between 0.7 and 1.2. In seven experiments like the one in Table 2, considerably greater PI values (mean ± SEM = 2.4 ± 0.5), approaching those found in naturally lactating mice, were seen. These results demonstrate that exogenous hormones can induce the mammary glands of virgin mice to behave like those of naturally lactating females in acting as homing sites for MN lymphoblasts. Hormone treatment did not alter the natural capacity of the small intestine to act as a homing site for MN lymphoblasts (7) and had no effect on homing of either PN or MN lymphoblasts to various lymphoid organs (not shown).

Influence of Testosterone on Homing of MN Lymphoblasts to Mammary Glands of Lactating Mice. The converse of the preceding experiment was to determine whether testosterone, which inhibits development of the secretory immune system in the mammary gland (Table 1), would inhibit homing of MN lymphoblasts to the mammary glands of lactating mice. When lactating mothers were treated with schedule 10 (Fig. 1) begun 4 days post partum, a PI of 0.6 was obtained. This value is within the range found in virgin mice. The same donor MN cells migrated efficiently to the mammary glands of lactating mice not given exogenous hormones. Again, the hormone treatment had no effect on homing to the small intestine.

(*B*) Untreated 8-week-old virgin showing an occasional IgA plasma cell (arrow). (*C*) Untreated 20-day lactating mouse. The glands are fully developed and distended with milk; flattening of the lining epithelial cells is thought to result from increased intraluminal pressure. (*D*) Untreated 20-day lactating mouse. Plasma cells (e.g., at arrows) are evident, and IgA is also visible in the flattened epithelium. (*E*) Virgin treated with progesterone, estrogen, and prolactin (schedule 7). There is marked proliferation of glandular and ductal epithelial cells (cf. *A*). (*F*) Virgin treated with progesterone, estrogen, and prolactin (schedule 7). IgA plasma cells (focal, bright corpuscles) are conspicuous. The glandular elements, as shown in *E*, show diffuse fluorescence due to intraepithelial IgA.

DISCUSSION

It has long been clear that multiple hormones are necessary for optimal development of the epithelial elements of the mammary gland (6, 8–12). The most important hormones appear to be estrogen, progesterone, and prolactin (8, 9); however, other hormones such as cortisone (6) have been found to have auxiliary effects. Our findings show that development of the secretory immune system in the mammary gland is also controlled by hormones, presumably through their direct or indirect action on nonlymphoid elements of the gland. It is important to stress that the hormone effect is specific for immunocytes making IgA. As in naturally lactating mice (1), in the animals given hormones in the present experiments the increase in plasma cells was similarly selective.

Of the hormones given singly to virgin females, prolactin had the greatest effect on number of IgA plasma cells and intraepithelial IgA. For maximal effect, at least three hormones—progesterone, estrogen, and prolactin—were necessary. This combination produced a striking elevation in IgA plasma cell numbers over those seen in the mammary glands of control virgin mice, an elevation comparable to that seen at term pregnancy. In contrast to progesterone, estrogen, and prolactin, testosterone started early in natural lactation inhibited both the development of glandular tissue and the infiltration of IgA plasma cells. In males, the mammary glands, although normally undeveloped, apparently have the potential for developing a full secretory immune system. For this to be demonstrated, the antagonistic effect of endogenous androgen had to be eliminated.

In all our experiments, development of glandular elements, increases in intraepithelial IgA, and increases in the number of plasma cells took place in parallel. Similarly, involution of the glandular elements was associated with declines in the number of plasma cells and the intraepithelial content of IgA. These fluctuations in plasma cell numbers, whether produced naturally during and after lactation (1) or artifically by hormone administration, were invariably accompanied by a corresponding increase or decrease in the propensity of lymphoblasts from MN to home to the prepared gland. Such homing is always selective for the precursors of IgA plasma cells as opposed to those making other classes of immunoglobulin (2). Others (13) have found that, in uterine secretions, both IgA and IgG levels may be increased after stimulation with sex hormones; the source of the immunoglobulins in this case is unknown. Our findings tend to implicate recruitment of GALT-derived precursors by a hormone-dependent mechanism as the main process by which plasma cells increase in the mammary gland. However, it is quite possible that further division of precommitted IgA precursor cells or induction of uncommitted B cells also takes place *in situ* under hormonal influence.

The origin of IgA plasma cells in the lamina propria of the intestine is also thought to be in the GALT (reviewed in refs. 14 and 15). The precursors arise in the Peyer's patches and migrate to the MN and thence via the circulation to the lamina propria, thus participating in an IgA cell cycle. The precursors of the IgA plasma cells in the mammary gland also appear to originate in this pool and have similar properties,—i.e., they are lymphoblasts that bear endogenous surface IgA and lack surface IgM and IgG and complement receptors (2). We speculate that, if the precursor populations are indeed the same, it is likely that homing involves expression in both mammary gland and intestine of the same receptor capable of interacting with a particular element on the surface of an IgA immunoblast. The nature of both the tissue receptor and the immunoblast element is unknown at present. The tissue receptor could be constitutively expressed on the surface of many cells in the intestine and on a small contingent of cells in the resting mammary gland, or it might be a secreted product of these cells and act as a chemotactic factor. Expansion of the population of receptor-bearing (or secreting) cells under the influence of hormones would explain the increased attraction of the lactating mammary gland for homing B cells. Alternatively, receptors in the mammary gland might be synthesized *de novo* under the influence of hormones. Inducibility of the tissue receptor by exogenous hormones affords an approach to its isolation and the study of its interaction with IgA plasmablasts.

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